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PROTEIN AND NUCLEIC ACID SYNTHESIS IN SUBCELLULAR FRACTIONS OF BACTERIAL CELLS[1]

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1. INTRODUCTION

It is now widely accepted that significant advances in our understanding of protein synthesis requires the development of *in vitro* systems permitting a direct experimental analysis. As a consequence, recent years have witnessed an increasingly intensive search for methods designed to isolate cell components capable of carrying out reactions relevant to the fabrication of the biologically specific macromolecules. The almost incredible optimism which characterized the initiation of such projects has been justified with breathtaking speed.

In illustration, one need merely mention the pioneering experiments of Gale (1955), the more recent accumulation of data on amino acid activation (Hoagland, 1955; DeMoss and Novelli, 1955; Novelli, 1958; Lipmann, 1958) and their transfer via the soluble RNA fraction[2] (Hoagland *et al.*, 1957; Ogata and Nokara, 1957; Schweet *et al.*, 1958; Berg and Offengand, 1958; Lipman, 1958). To these must be added the astonishing advances in our understanding of nucleic acid polymer synthesis we owe to the efforts of Ochoa and his group on RNA (Grunberg-Manago *et al.*, 1956; Ochoa and Heppel, 1956) and of the Kornberg (1956) school on DNA. The last two investigations, in particular, pose an interesting question of methodology for all investigators in these and contiguous fields. In both instances, the synthesis of the relevant macromolecules were obtained with highly purified enzyme preparations, an achievement one would have thought on *a priori* grounds would be reached in the distant future. However, this having been accomplished, does it make any sense to continue the study of cruder preparations? In answering this question, one must immediately grant the obvious

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[2] The following abbreviations are used: RNA, ribonucleic acid; DNA, deoxyribonucleic acid, RNAase and DNAase for the corresponding nucleolytic enzymes; PRN for polyribonucleotide composed of ribonucleotide subunits with no implications of its relation to normal "RNA"; ATP, GTP, CTP, UTP, for adenosine, guanosine, cytidine and uridine triphosphates respectively. The corresponding diphosphates will be denoted by ADP, GDP, etc. Tris, tris (hydroxymethyl) amino methane.

advantages which stem from the use of a system purified to a level of simplicity which permits unequivocal interpretation of data derived from its use. There is, however, the danger that in efforts at purification the system may be simplified to the point where it will no longer possess properties of importance for the solution of other central biological issues. Of particular pertinence here is the problem of the interrelation amongst the biosynthetic mechanisms of RNA, DNA and protein. It is true that in these days of unlimited optimism, one can justifiably hope that such interrelations will be revealed by reconstructive additions of the purified systems as we know them today. This does presuppose that they contain all the necessary components, an assumption for which no guarantee can at present be offered. In its absence it would seem desirable to continue some investigations with preparations which have not been carried to the state of purity which characterizes the goal of the enzyme chemist. It was our feeling that the latter approach was being so vigorously and effectively prosecuted in other laboratories that ours could add little to these efforts. It seemed likely that a useful function could still be served by continuing the study of systems which were sufficiently crude to retain the inherent potentialities of exhibiting interrelations amongst the three macromolecules of major interest. To these more philosophical justifications must be added the more practical one that no purified enzyme system has thus far been isolated which can synthesize protein molecules.

Before embarking on a brief description of our most recent efforts it may be of interest to summarize briefly the evolution of the *in vitro* system which at present engages our major attention.

Our primary interest from the outset was the development of a cell-free system capable of carrying out whatever sequence of reactions might be necessary for the ultimate production of a recognizable protein molecule.

Our search for such a preparation began with the bacterial protoplast system described by Weibull (1953). The osmotic fragility of the protoplast promised to provide a uniquely suitable departure point for the derivation of subcellular fractions by procedures far gentler than any required to disrupt intact bacterial cells. All the initial exploratory experiments were performed with protoplasts of *B. megaterium*. Subsequently, methods of achieving similar preparations with *E. coli* were devised (Lederberg, 1956; Repaske, 1957; Zinder and Arndt, 1956) and our attention was turned to this organism in view of the wealth of genetically marked strains available in this form. All the experiments to be detailed in the present report were performed with material obtained from protoplasts of *E. coli*.

The first successes in observing synthetic activity were obtained with total lysates of *B. megaterium*. These preparations could fabricate enzymatically active protein and both types of nucleic acid. Extensive investigations

were made to determine the optimal conditions and supplementation required by the lysates to exhibit maximal activities. These results have been previously summarized (Spiegelman, 1956). The information gained was extremely useful for later studies since many of the properties and requirements exhibited by the lysates of *B. megaterium* were also possessed by the similar preparations prepared from *E. coli*.

Once reproducibility was achieved, it became clear that the crude lysates were too complex and heterogeneous to permit the performance of experiments readily interpretable in terms of either known cellular components or defined enzymatic reactions. As prepared, lysates were mixtures of soluble enzyme systems, ribonucleoprotein particles and membrane fragments of various sizes. Further progress demanded the evolvement of procedures which would separate these different components and so permit an identification of which ones were necessary and responsible for the synthetic functions being studied. After a large number of exploratory experiments which tested a variety of conditions, a reproducible procedure was ultimately developed which permitted the separation of defined fractions possessing synthetic activities of interest. It is the purpose of the present paper to describe the preparation and properties of these fractions and summarize the information obtained in the course of these studies with particular emphasis on those aspects which are relevant to the problem of the relation between nucleic acid and protein synthesis.

2. THE FRACTIONATION OF OSMOTIC LYSATES

As a necessary prelude to a discussion of the chemical and synthetic properties of the fractions studied we begin with a brief description of the methods employed in their isolation. In what follows attention is confined to *E. coli* protoplasts prepared by the Lederberg penicillin procedure (1956). After harvesting by centrifugation, the protoplasts are washed with 10% sucrose buffered with 0.05 *M* Tris at pH 7.4 and supplemented with 1×10^{-3} *M* $MgCl_2$. They are then resuspended in 9 % glycerol containing Tris and $MgCl_2$ at the levels just noted, frozen and stored at $-25°C$. The density of protoplasts in the freezing mixture is 40 times that which obtains at the time of harvesting. If kept in the frozen state they retain their activity without detectable loss for at least 3 months.

When needed, tubes containing the desired amount of protoplasts are removed, thawed and the protoplasts recovered by centrifugation. To attain uniformity, differences which may exist from one batch of protoplasts to another are randomized by choosing tubes from different dates to provide the material for a given experiment. The use of this procedure has yielded results with satisfactory reproducibility.

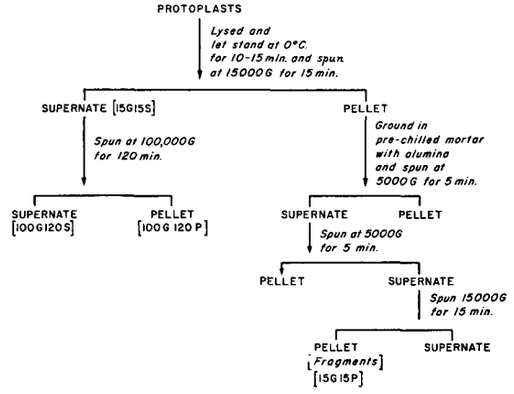

FIG. 1. Flow sheet of centrifugal fractionation of total lysates.

Exposure of protoplasts to a medium lacking an osmotic stabilizer (e.g., sucrose) leads to extensive lysis. The resulting lysates provide the starting material for the preparation of the various fractions of interest. The procedure followed in the centrifugal fractionation is depicted as a flow diagram in Fig. 1. Lysis is accomplished by exposing the pellet from 5 ml of protoplasts to 5 ml of 0.05 *M* Tris buffer (pH 7.4) containing 1×10^{-3} *M* $MgCl_2$ and 5×10^{-3} *M* $MnCl_2$. This is followed by a centrifugal separation at 15,000 *g* into a low-speed pellet and a supernate fraction (15G15S). The Mg concentration of the supernatant is raised to 1×10^{-2} *M* and it is then further separated by high-speed centrifugation into a 100G120S fraction, containing the bulk of the soluble proteins, and a pellet fraction (100G120P).

The low-speed pellet corresponds essentially to the fraction designated as the "shockate" in the earlier investigations (Spiegelman, 1956). It contains membrane fragments of various sizes. Grinding this pellet with a small amount of alumina (ca. 1/2 wet weight of the pellet) converts it into a prepa-

TABLE 1. *Composition of centrifugal fractions.*

Fractions obtained as in Fig. 1. Nucleic acid analyzed on hot acid hydrolysate by dichromatic readings on the UV, the orcinol reaction (Dische and Schwartz, 1937) and Burton's (1956) modification of the Dische reaction. Over 80% of the DNA is lost as acid-precipitable polymer during lysis. 10% of the nucleic acid in the 15G15P fraction is DNA. Protein was analyzed by the Lowry *et al.* (1951) modification of the Folin reaction.

Fraction	% of total NA	% of total Protein
15G15P	6	8
100G120P	80	36
100G120S	14	56

ration from which relatively pure and uniform membrane material can be readily obtained in fair yield. The grinding step serves two functions. It ensures a virtually complete disruption of any comparatively intact material and in addition fragments the membranes to sizes which do not pellet at low speeds. After the grinding, the material is resuspended in lysing medium

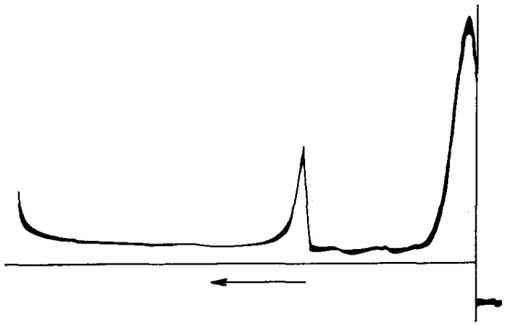

FIG. 2. Ultracentrifuge pattern of 15G15S fraction. Centrifugation at 59,740 rpm in a Spino Analytical Centrifuge. Under conditions described in text only one major particulate component is observed.

and subjected to two successive clearing spins at 5000 *g* which remove the alumina and any residue of large fragments. The remaining supernatant is centrifuged at 15,000 *g* for 15 minutes to yield the membrane 15G15P fraction. The latter is routinely subjected to at least one wash with lysing medium prior to use or analysis.

Table 1 summarizes the distribution of protein and ribonucleic acid in the three fractions. It will be noted that the bulk of the RNA is found in the high-speed pellet although some appears in both the high-speed supernate and membrane fractions. In the case of protein the picture is somewhat reversed in that the bulk of the protein is found in the high-speed supernate.

It may be useful to append here a few details concerning the reproducibility of the chemical and morphological characteristics of these fractions. It must be emphasized that the distribution of components summarized in table 1 is characteristic of the fractions only if they are isolated from the material and by the procedures described. Introducing any of the methods usually employed in disrupting intact cells (e.g., sonication or grinding with 2–3 times the wet weight of alumina) has in our hands led to preparation possessing markedly different chemical and biosynthetic properties. Further, unless the Mg level is maintained at a high level (0.01 *M*) during the isolation the RNA content of the 100G120S can rise to between 25 and 35 per cent of the total. Such modifications in the distribution of the chemical constituents are accompanied by changes in the structure of the 100G120P fraction.

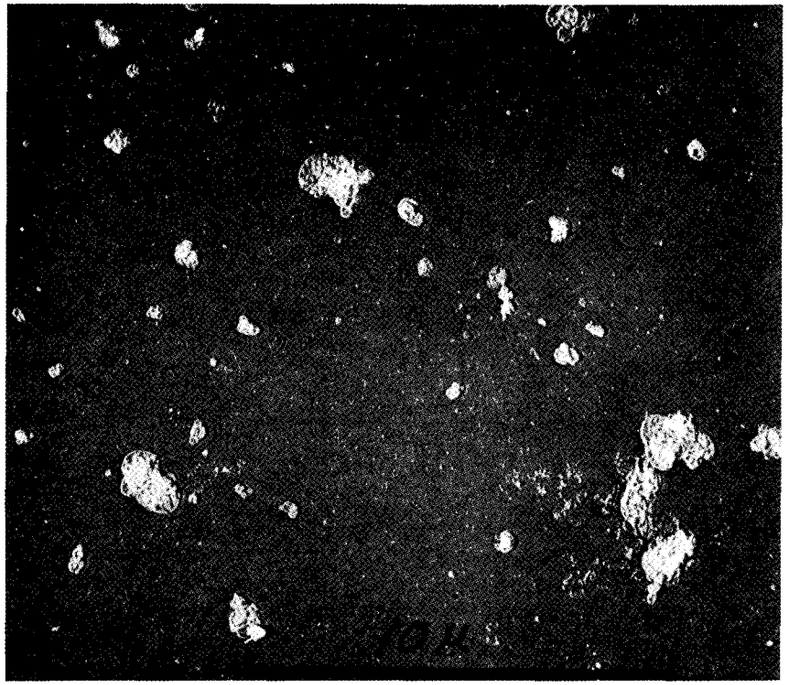

FIG. 3. Electron photomicrograph of membrane fraction.

Electron microphotographs of the 15G15S fractions prepared in the presence of 0.01 *M* $MgCl_2$ show spherical bodies of predominantly one size. The uniformity of these preparations is illustrated by the ultracentrifuge pattern shown in Fig. 2. Aside from the soluble proteins there is a single dominant peak of material corresponding to an 70S component. The 70S particles can be reversibly broken down into 30S and 50S components by dialysis against 0.1 *M* phosphate buffer at pH 7.4. Dialysis of the resulting mixture against 0.01 *M* $MgCl_2$ buffered with 0.05 *M* Tris at pH 7.4 results in what appears to be a complete reassembly as evidenced by the disappearance of the 30S and 50S peaks and the reappearance of the 70S component (Spiegelman, 1958).

Comparative studies carried out in our laboratory with protoplasts as the starting material suggests that much of the confusion existent in the literature on the particulate composition of *E. coli* cells may be ascribed to the variety of ionic mixtures and methods of cell rupture used by different authors in the preparation of extracts for centrifugal analysis.

Fig. 3 is an electron microphotograph of the 15G15P fraction and illustrates the heterogeneous mixture of fragment sizes of which it is comprised.

TABLE 2. *Molar ratios of RNA in fractions.*

Fractions obtained as in Fig. 1. Analysis of bases by electrophoretic separation of nucleotides (Davidson and Smellie, 1952).

Fraction	Cytidylate	Adenylate	Guanylate	Uridylate
100G120P	21	21	38	20
100G120S	20	20	30	30
15G15P	18	23	34	25
Total	21	21	37	21

None of them possess the electron density of a protoplast. When prepared under the standard conditions described, the chemical composition of this fraction is uniform and reproducible. It may be noted that, if fresh, rather than glycerol-frozen protoplasts are used, consistently higher RNA to protein ratios are obtained (Ben-Porat, 1958).

The method employed in their separation and constant composition gave reason to hope that the three fractions described were distinct entities which had some relevance to the structure of the cells from which they were derived. Certain distinguishing features served to strengthen this supposition. Thus, the particle fraction of both *E. coli* (Spiegelman, 1958) and *B. megaterium* (Aronson and Spiegelman, 1958*a*, 1958*b*) are rich in basic proteins characterized by solubility in dilute mineral acid. Further, compared to the other fractions, the particle proteins are extremely poor in sulphur containing amino acids, corresponding to less than 5 % of that characteristic of the bulk of the proteins of the cell. According to the analysis of Roberts, Britten and Boton (1958) no cysteine is detectable in particle proteins.

Table 2 shows that the base composition of the RNA also serves to distinguish the three fractions (Seaman and Spiegelman, 1958). Both the particle and membrane fractions are comparatively rich in guanylate. However, the uridylate ratio is higher in the 15G15P fraction. The soluble 100G120S fraction possesses the lowest molar ratio of guanylate and the highest relative uridylate content. It is the only component containing RNA in which the sum of the purines is equal to that of the pyrimidines. These differentiating features of base composition disappear when the fractions are isolated by procedures involving sonication or low Mg levels in the environment.

3. THE BEHAVIOUR OF THE FRACTIONS *IN SITU*

Before considering the capacity of the three fractions described to synthesize protein and nucleic acid *in vitro* it is of some interest to examine their behaviour in the intact cell.

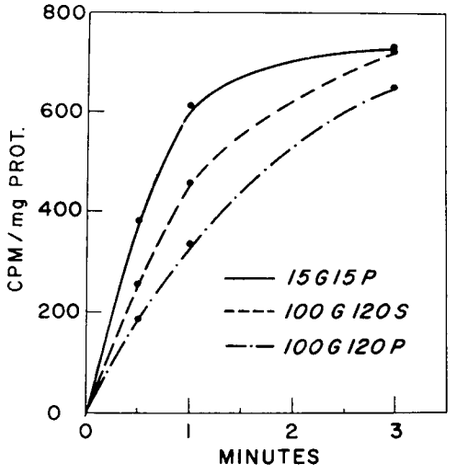

FIG. 4. Incorporation of C^{14}-leucine in intact protoplasts. Fractionation according to diagram of Fig. 1; membranes (15G15P), soluble proteins (100G120S), ribonucleoprotein particles (100G120P).

Fig. 4 shows the results of a representative short-term labeling experiment with C^{14}-leucine. Intact protoplasts were exposed to labeled amino acid for the periods indicated, synthesis being stopped by chilling and the use of NaN_3. The samples were then centrifuged and the protoplasts fractionated as described in Fig. 1. It is evident that the 15G15P fraction is the one most rapidly labeled, followed by the soluble proteins and finally by the particulate components.

An analogous experiment (Ben-Porat, 1958) designed to obtain the same sort of information with respect to RNA metabolism is summarized in

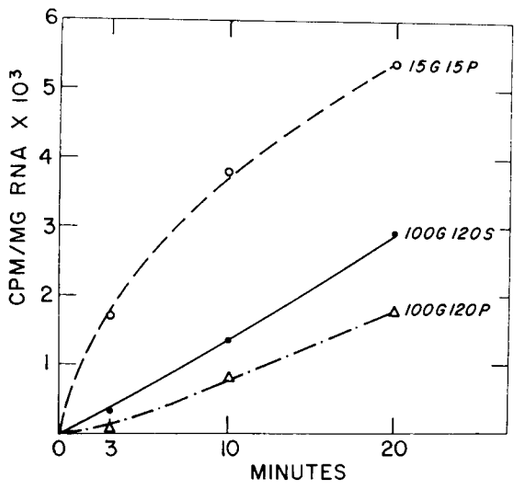

FIG. 5. Incorporation of C^{14}-uridine in intact protoplasts. Fractionation according to diagram of Fig. 1 to yield membranes (15G15P), soluble proteins (100G120S), ribonucleoprotein particles (100H120P). Numbers represent thousands of counts per minute.

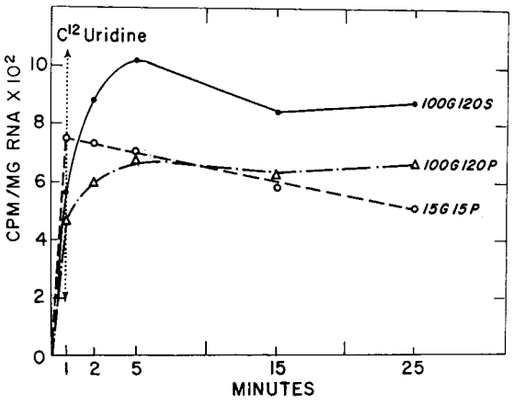

FIG. 6. Pulse experiment with C^{14}-uridine in intact protoplasts. Fractionation according to diagram of Fig. 1 to yield membranes (15G 15P), soluble proteins (100G 120S) and ribonucleoprotein particles (100G120P). At one minute C^{14}-uridine diluted with C^{12}uridine. Numbers represent hundreds of counts per minute.

Fig. 5. Here C^{14}-uridine was used and a similar fractionation carried out. Again, we see that it is the membrane fraction which is the most rapid in macromolecular synthesis.

The results obtained with C^{14}-uridine are in qualitative agreement with the experiments of Volkin and Astrachan (1956*a*, *b*) on the distribution of P^{32} in phage-infected bacteria. This assumes that we can equate our 15G15P with their P_1 fraction.

Other experiments (Ben-Porat, 1958) have analyzed the flow of labeled material in pulse experiments and one of these is summarized in Fig. 6. Protoplasts were exposed to C^{14}-uridine for one minute, at the end of which a sample was removed for fractionation and specific activity determination. To the remainder an amount of C^{12}-uridine was added sufficient to achieve a 100-fold dilution of the label. Samples were then removed at the time periods indicated to determine the subsequent flow of the material labeled in the first minute. It will be noted that in the period of labeling the 15G15P fraction achieved the highest specific activity. Following dilution of the label, the activity of the membranes falls while the other two fractions are rising.

Such results are consistent with the interpretation that the membrane fraction is concerned with the initial act of fabricating RNA macromolecules which ultimately are transferred to the soluble and particulate fraction.

4. AMINO ACID INCORPORATION INTO FRACTIONS ISOLATED FROM OSMOTIC LYSATES

From the experiments described in the preceding paragraphs, as well as others not detailed, one is led to conclude that the principal site of protein and RNA synthesis is physically associated with the protoplast membranes.

On the basis of such evidence, one would be led to predict that attempts to isolate subcellular fractions possessing the ability to fabricate complete protein molecules are most likely to succeed if attention is focused on the membrane fraction.

A comparative study was made of the abilities of these three fractions to carry out the various steps which are presumed to constitute recognizable stages in protein synthesis. Particular attention was paid to the features mentioned in the introductory paragraphs. These included the ATP dependent carboxyl activation mediated by the amino acid activating enzymes, the presence of the soluble RNA acceptor and, finally, the formation of peptide bonds. To distinguish between the last two, advantage was taken of the fact that amino acids fixed to the soluble RNA fraction are stable to cold acid, but are liberated by exposure to hot acid (cf. Berg and Offengand, 1958). Finally, an examination was always made of the response of the incorporation of any given amino acid to supplementation with a complete mixture of the others. It was felt that a positive response represented a good diagnostic indication that the reaction being studied was more likely to represent the formation of a complete or nearly complete protein molecule.

(*a*) *The high-speed supernate* (*100G120S*).

The high-speed supernatant fraction contains the major portion of the amino acid activating enzymes as measured by the pyrophosphate exchange reaction (DeMoss and Novelli, 1955). As may be seen in Fig. 7 it can carry out a reaction resulting in the fixation of amino acids into a linkage which is stable to cold, but not to hot acid. It is further noteworthy that the amino acids are incorporated into a linkage which is highly labile under the conditions of the incubation. Direct evidence has been obtained that the amino acids fixed in the 100G120S fraction are extremely sensitive to RNAase by the following sort of experiment. The reaction was run for 5 minutes and then stopped by the addition of 2.5 volumes of cold alcohol and 10^{-2} *M* $MgCl_2$. After precipitation was complete, the precipitate was recovered and washed several times. The pellet was then redissolved in Tris buffer at 7.5 and one aliquot was exposed to 100 μg RNAase per ml for 5 minutes. Treated and control preparations were then precipitated with cold 10 % TCA, the pellets washed with cold acid and then dissolved and counted. The RNAase-treated material contained no detectable counts as compared to a 75 % recovery of the counts in the untreated control.

Maximal amino acid fixation in this system requires the presence of ATP and 5′-ribotides. The reaction is not inhibited by even elevated levels (500 μg/ml) of chloramphenicol. The fixation of a given amino acid is not augmented by the presence of a mixture of others. Finally, it may be noted that no combination of supplements in the form of various intermediates

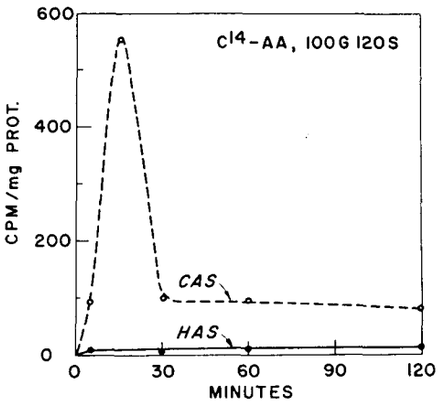

FIG. 7. Incorporation of C^{14}-leucine into isolated soluble protein fraction (100G120S). Counts stable to cold acid (CAS); counts stable to hot acid (HAS).

served to confer on this fraction the ability to insert amino acids into linkages stable to hot acid.

Insofar as amino acid incorporation is concerned, the 100G120S fraction possesses features which previous authors (Hoagland *et al.*, 1957; Lipman, 1958; Berg and Offengand, 1958) have reported as characterizing the "soluble RNA" acceptor system.

(*b*) *The high-speed pellet fraction* (*100G120P*).

The particulate fraction, prepared as described, contains amino acid activating enzymes. It can incorporate labeled amino acids into linkages stable to both hot and cold acids. Comparison of the relevant curves in Figures 6 and 7 reveals that fixation in the linkage stable only to cold acid (CAS) precedes in time the appearance of label in hot acid stable (HAS) form. Thus, in 15 minutes, incorporation of CAS counts is complete. At that time only half the counts are retained after hot acid extraction. During the next 15 minutes all the counts precipitable with cold acid are converted into a form which is stable to hot acid.

Optimal incorporation requires the presence of ATP and 5′-ribotides. The incorporation of any given amino acid is uninfluenced by supplementation with a complete mixture of the others. Neither is it affected by the presence of chloramphenicol. Again, the activity observed and its properties do not encourage the belief that one is here studying a system capable of carrying all the stages of protein synthesis.

(*c*) *The low-speed supernatant* (*15G15S*)

This fraction is the one which yields the 100G120P and 100G120S fractions by high-speed centrifugation. It is, therefore, a combination of the

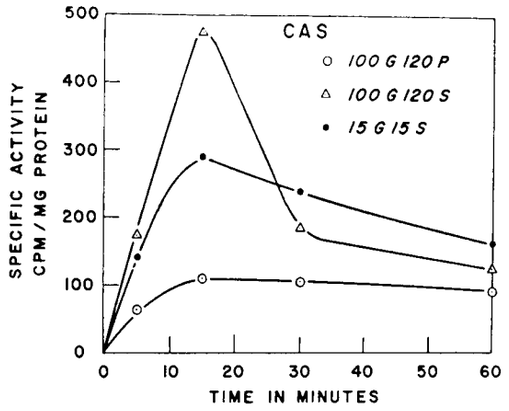

FIG. 8. Incorporation of C^{14}-leucine into cold acid stable (CAS) linkage in isolated fractions.

two fractions we have discussed in detail in the two preceding sections. Its amino acid incorporating abilities are, not surprisingly, a composite of the high-speed pellet and supernate fractions. The CAS fixing abilities of the three fractions all derived from the same preparation are given in Fig. 8. The corresponding kinetics of incorporation into hot acid stable linkages are shown in Fig. 9. Quantitatively the 15G15S fraction is somewhat more active than one would expect from adding the observed activities of its components functioning in isolation. Nevertheless, like them, the incorporation of a particular amino acid shows no response to supplementation with a complete mixture of the others. Neither does it indicate by any other property that it is capable of manufacturing complete protein molecules.

Considering only the data derived from examination of hot acid stable counts, the extent of the activity observed is discouragingly small in all of the fractions described thus far. In no case did the "synthesis" correspond

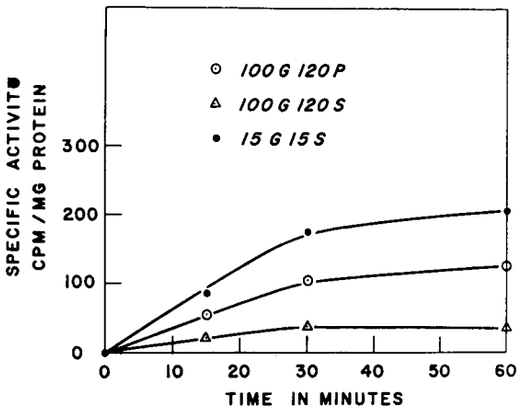

FIG. 9. Incorporation of C^{14}-leucine into hot acid stable (HAS) linkage in isolated fractions.

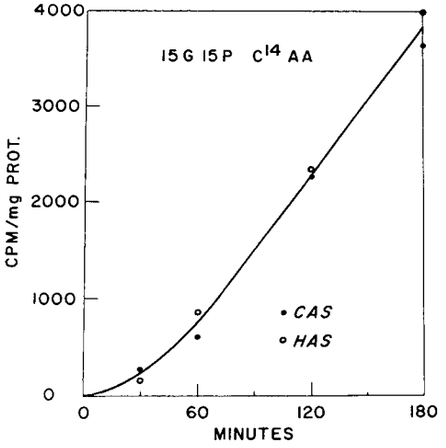

FIG. 10. Incorporation of C^{14}-leucine into isolated membrane fraction. Open circles represent counts stable to hot acid, and closed circles counts stable to cold acid.

to more than 0.1% of the protein input and in many instances it was considerably less. If any of these fractions contain active protein synthesizing mechanisms, our procedures have failed to reveal them. In view of the success attained with the next fraction to be described, one is tempted to conclude, at least tentatively, that the comparative inactivity of the other fractions reflects a deficiency in the preparations rather than in the methods employed in their isolation and the supplementation used to test them.

(d) The membrane fragment preparation (15G15P)

The membrane fragments were the only fraction found to possess significant capacity to synthesize protein as measured by either incorporation or induced enzyme synthesis. This is a finding we previously suggested might have been expected from the experiments which compared the activities of the three fractions in the intact protoplast.

Like all the other fractions examined, preparations of membrane fragments contain enzymes capable of carrying out a pyrophosphate exchange reaction with ATP in the presence of amino acids. These activities are retained even after several washings. It must, however, be noted that the bulk of these activities are found in the 100G120S fraction.

The amino acid incorporating activity of the membrane fraction exceeds by a factor of 100 that observed with the other fractions when examined under comparable conditions. Fig. 10 shows the kinetics of incorporation with a membrane preparation and compares insertion into linkages stable to hot and cold acid. Unlike the high-speed pellet and supernatant fraction there is here no observable incorporation of CAS counts which are not also stable to hot acid.

It will be noted further that the kinetics of the incorporation is very diffe-

rent from that seen with the other fractions. There is a slight lag at the onset followed by a linear rate of synthesis which continues for more than 3 hours in the vast majority of preparations examined. The specific activity attained at the 3-hour point corresponds to 15% synthesis based on the protein input.

A variety of combinations of various nucleic acid intermediates were tested for their effect on this incorporation in an attempt to achieve an optimal mixture. The experiment described in Fig. 10 was carried out under optimal conditions of supplementation detailed in table 3 which compares the effects of removing various components on incorporation of C^{14}-leucine. The omission of either ATP or the 5′-nucleotides results in drastic loss of the incorporation observed over a 2.5-hour period. Mn appears to be a mandatory requirement, an observation made in our earlier experiments with crude lysates of *B. megaterium* (Spiegelman, 1956) and *E. coli*. It will be further noted that the presence of other amino acids is necessary if any significant incorporation of leucine is to be observed. The same situation was obtained with other labeled amino acids tested. This dependence of incorporating activity on the presence of a virtually complete mixture of amino acids serves to distinguish the incorporation observed in this fraction from the others described. Another unique property noted in table 3 is its sensitivity to chloramphenicol. None of the other fractions are inhibited by this agent.

One other finding, relating to the lag apparent in Fig. 8, may be noted. An attempt to identify the cause of this delay led to the discovery that it could be eliminated by including the other three riboside triphosphates. The corresponding diphosphates had no detectable effect on the extent or kinetics of amino acid incorporation.

TABLE 3. *C^{14}-Amino acid incorporation into membrane fraction 15G15P.*

Complete incubation mixture contains per ml: 20 μM KCl; 50 μM Tris; 50 μM maleate; pH 6.5; 5 μM Mn; 1 μM Mg; four 5′-ribotides, 0.1 μM each; four 5′-deoxyribotides, 0.1 μM each. Amino acids mixture in ratio corresponding to *E. coli* protein, 2 mg. ATP 2 μM between 100–200 μg of enzyme preparation. Counting done on automatic micromil gas-flow counter, Nuclear-Chicago.

Medium	Incorporation mμM AA/mg prot.	% of complete
Complete	600	100
− ATP	120	20
− 5′-ribotides	220	37
− 5′-deoxytides	200	33
− (5′-ribotides + 5′-deoxytides)	100	17
− Other amino acids	20	3
+ Chloramphenicol (200 μg/ml)	40	7
− Mn (5×10^{-3} M)	0	0

5. POLYRIBONUCLEOTIDE SYNTHESIS

The supplemental responses of amino acid incorporation yielded persistent evidence for the active involvement of nucleic acid metabolism. Particularly interesting was the finding of consistent stimulation which occurred on the addition of the 5′-deoxytides. These observations are reminiscent of the preliminary reports of Beljansky (1954) and Lester (1953) who observed stimulation of incorporation in total lysates on the addition of DNAase. Such results suggested the intriguing possibility that we might here ultimately have the opportunity of studying the interaction between the synthetic mechanisms of the two types of nucleic acid. However remote, such a possibility seemed worth exploring, for, despite the startling advances recorded in recent years, the direct experimental analysis of the biosynthetic interrelations between DNA and RNA has thus far remained an unexplored aspect of the central problem.

Indications of active polyribonucleotide metabolism was searched for employing both net increments of acid-precipitable nucleic acid polymer and the incorporation of P^{32}-labeled 5′-ribonucleotides. Evidence was quickly obtained indicating that both the 15G15S and 15G15P fractions possessed extensive synthetic activities worthy of further analysis.

Many features of these systems are still under investigation and it is difficult at the present time to specify with certainty which are relevant and which are biochemical artefacts reflecting the comparative crudity of the preparations being studied. We summarize here those data which illustrate the possible potentialities of these systems as tools for the further investigation of certain problems of obvious interest.

Because of our previous experience, much of our initial efforts were concentrated on the 15G15P fraction. Consequently, we here detail only the experiments performed with it.

(*a*) *Stabilization of polyribonucleotide synthesized*

Preliminary experiments with the membrane fraction made it quickly evident that polyribonucleotide synthesis could be readily studied in terms of net increases of acid-precipitable polymer. There was, however, at the beginning a disturbing amount of inconsistency and poor reproducibility. Kinetic analysis suggested that this was due to a degradation competing with the synthetic reaction leading under certain circumstances to loss of the product. A variety of conditions and agents were tested in an effort to overcome this difficulty. The attempt was either to inhibit the nucleolytic enzymes present or introduce a substance which would combine with the polymer and thus protect it against destruction. Ultimately, it was found that the use of the polyamine, spermine, at a concentration of 5×10^{-3} M,

TABLE 4. *Effect of supplements on PRN synthesis in 15G15P.*

Complete system contains per ml: 5 μM Mn; 1 μM Mg; 20 μM KCl; 50 μM Tris; 50 μM of maleate at pH 6.5; 0.2 μM of each 5′-ribotide and deoxytide; 2 μM ATP; 0.4 μM of each of the other riboside triphosphates; 5 μM spermine; ca. 200 μg protein of enzyme preparation containing 20 μg RNA. Analysis for PRN as in Table 1.

Incubation	Δ PRN in μg/mg prot.	% Change	% of complete
Complete	340	+380	100
−ATP	0	0	0
−Riboside triphosphates (TOP)	70	+ 80	21
−TOP; +riboside diphosphates	120	+130	34
−(ribotides and deoxytides)	10	+ 11	3
−Mn	0	0	0

provided the protecting effect which was sought. Fig. 11 shows the difference of synthetic activity observed in the presence and absence of this agent at two levels of substrate input. In the absence of spermine, synthesis of polyribonucleotide occurs for 90 minutes followed by loss of the product. If excess substrate is provided, the presence of spermine insures a linear synthesis for at least three hours. It should be noted that at the three-hour point the synthesis corresponds to over a 10-fold increase of the input of polyribonucleotide.

(*b*) *Nutritional and ionic requirements for polyribonucleotide synthesis.*

With this problem resolved, it became possible to inquire into the conditions and supplements required for optimal synthesis of polyribonucleotide.

The pH optimum for the synthesis was found to be between 6.0 and 6.5 with a plateau in this range. In view of the effects observed with the ribose triphosphates on amino acid incorporation, they were compared with corresponding diphosphates in the system synthesizing polyribonucleotide. The results observed were clear and consistent. The ribose-disphosphates were definitely inferior and their use was invariably attended by a lag period of 1 hour before any significant polyribonucleotide synthesis was observed. In contrast, as may be seen from Fig. 10, synthesis in the presence of triphosphates begins immediately. Another property which was in agreement with the information gathered during the study of amino acid incorporation was the finding that the polyribonucleotide formation had a mandatory requirement for Mn. The optimal level of this ion was found to be 1×10^{-3} M. These and other characteristics of the system are summarized briefly in table 4.

It will be noted that ATP is required and must be present at levels exceeding 0.5 μM per ml. While excellent synthesis can be observed with

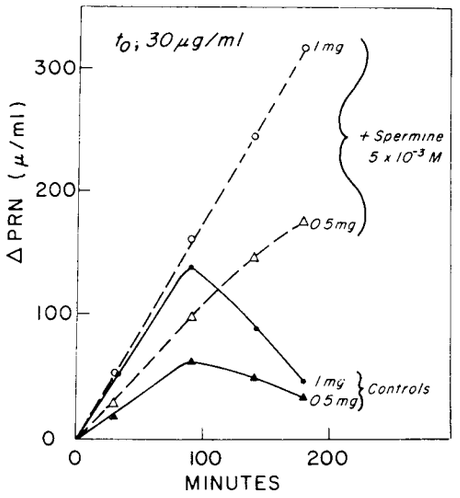

FIG. 11. Polyribonucleotide synthesis in membrane fraction. The input of nucleic acid was 30 μg/ml. The numbers beside the curves indicate the levels of ATP per ml. All were supplemented with the other three triphosphates at a level of 200 μg each per ml. The dotted curves represent synthesis in the presence of spermine.

1 μM of ATP per ml, somewhat better results are obtained with 2 μM per ml (see Fig. 11). The use of higher levels of ATP (4 μM/ml) leads to the formation of polyribonucleotides which possess the absorption spectra and resistance to RNAase digestion characteristic of poly-adenylate (see below).

The requirement for Mn was of particular interest in view of Littauer and Kornberg's (1957) studies on the ionic activation of the polyribonucleotide phosphorylase from *E. coli*. These authors found that Mn is unable to replace Mg as an activator. Indeed, it exerts a powerful (85%) inhibitory effect when included in the reaction mixtures at levels $\frac{1}{10}$ that of the Mg. It should be noted in comparison that PRN synthesis with an enzyme system prepared from *M. lysodeikticus* (Beers, 1957) is activated by Mn, although this ion is only $\frac{1}{3}$ as effective as Mg at optimal concentrations. It is difficult to interpret the significance of the Mn activation here since the Beers' preparation is capable of converting ADP to ATP and AMP.

TABLE 5. *Ionic requirements for PRN synthesis in 15G15P.*

Conditions of incubation same as "Complete" of table 4 except for indicated variation in ions. Incubation was for 1.5 hours.

Substrate	Mn (5×10^{-3} M)	Mg (1×10^{-3} M)	Δ (PRN)/mg prot.	% Increase
Triphosphates (all 4)	−	−	0	0
Triphosphates	+	+	70	100
Triphosphates	−	+	10	14
Triphosphates	+	−	135	197

Table 5 compares the effects of Mg and Mn, singly and in combination in the present system with triphosphates as substrates in an incubation carried out for 1.5 hours. It is clear that Mn is the preferred ion and that the further addition of Mg suppresses the amount of synthesis observed. The situation seems to be the reverse of that observed with the enzyme using the diphosphates as substrate.

(*c*) *The effect of nucleases on the synthesis of polyribonucleotide*

It was of obvious interest to examine the sensitivity of the synthesis to the presence of added RNAase and DNAase. In the experiments to be reported the incubations were carried out for 2 hours in the absence of spermine to avoid protection against enzymatic hydrolysis. The fragment preparations were pretreated with the indicated nuclease for 15 minutes at 30°C and then used without washing the enzyme away. All attempts to achieve reasonably complete removal of added enzymes have thus far failed. Each incubation contained, therefore, the relevant nucleolytic enzyme at the level given in table 6, which summarizes the results of two such experiments. It will be noted that the addition of either DNAase or RNAase results in a marked inhibition of PRN synthesis. The presence of the ribonuclease is accompanied by an 80 % loss of the input RNA.

Before considering the interpretation of these results, another feature of this system may be noted which is relevant to the question of its sensitivity to RNAase. If RNAase is exerting its inhibition by destroying the product, one would expect to be able to obviate this loss by encouraging the synthesis of a polyribonucleotide containing primarily purines, e.g., poly-adenylate. We have already mentioned that the present system carries out such a synthesis if the ATP is raised to 4 μM per ml or higher. Table 7 shows that, when the system is synthesizing PRN from ATP unsupplemented

TABLE 6. *Effects of nucleases on PRN synthesis in membrane fraction.*

Incubation with complete mixture of table 4, spermine omitted. Time of incubation 1.5 hours.

Incubation mixture Complete mixture	Δ PRN in μg/mg prot.	% Change	% of control
I. Control	340	380	—
I. DNAase (25 μg/ml)	30	33	8
I. RNAase (10 μg/ml)	−80	−88	<0
II. Control	416	460	—
II. DNAase (20 μg/ml)	0	0	0
II. RNAase (10 μg/ml)	−81	−90	<0

TABLE 7. *The effect of substrate on sensitivity to RNAase.*

Conditions of incubation same as "complete" of table 4; except for omission of spermine, and modifications noted below of Triphosphate additions.

Substrate mixture	RNAase 40 μg/ml	PRN synthesized in μg/mg prot.
ATP	−	350
ATP	+	280
ATP + CTP + UTP + GTP	−	470
ATP + CTP + UTP + GTP	+	0

by the other triphosphates, the accumulation of acid-precipitable polymer is comparatively insensitive to the presence of RNAase. The inclusion, however, of the other triphosphates leads to complete inhibition of the net synthesis.

Such data and those to be reported in the next section suggest that the presence of all four triphosphates leads to the formation of strands containing both purine and pyrimidine bases, rather than a mixture of strands each containing predominantly either purine or pyrimidine components.

(*d*) *The sensitivity of the product to nucleases and alkaline digestion*

The apparent sensitivity of the synthesis to DNAase was an interesting though puzzling finding and recalls the preliminary report of Hurwitz (1958) on the incorporation of cytidine riboside-triphosphate into a DNAase-sensitive, acid-precipitable polymer. If a mixed ribose-deoxyribose nucleic acid were being synthesized here, it might well be converted to acid-soluble oligonucleotides by either nucleolytic enzyme. Syntheses were consequently carried out on a scale large enough to permit isolation and partial purification by alcohol precipitation of approximately 20 mg of product. The resulting material was then exposed to RNAase, DNAase, and alkaline digestion under the conditions described in table 8 which summarizes the results obtained. The polymer is completely destroyed by RNAase and the KOH treatment. No detectable loss follows exposure to DNAase. With respect to the latter it may be noted that control reconstructions were run in which the PRN was deliberately contaminated with DNA. None of the added DNA was recovered following the DNAase treatment. At least by the criteria applied, there is no evidence for the belief that a significant number of deoxy-nucleotides are inserted into the ribonucleotide chains formed. The more sensitive examination by use of suitably labeled deoxytides remains as yet to be performed.

TABLE 8. *Properties of PRN formed in membrane fraction.*

Incubation for synthesis with "complete mixture" of table 4, spermine omitted. Product isolated by alcohol precipitation after 1.5 hours of incubation which resulted in a 4-fold increase in polyribonucleotide. Dissolved in Tris-buffer to contain finally 400 μg PRN per ml.

Treatment	Amount of acid-precipitable PRN in μg/ml after treatment	% of control
None	400	–
Incubation in 0.3 *N* KOH 37°C (15 hrs.)	0	0
DNAase 200 μg 10 min. 30°	410	102
RNAase 200 μg 10 min. 30°	0	0

(*e*) *On the nature of the reaction and its comparison with the phosphorylase enzyme.*

It is obvious from the description of the system that it is too crude at the present time to permit us to draw any conclusions relevant to the chemical mechanisms involved in the observed synthesis of polyribonucleotide. Many obvious experiments remain to be done when the appropriately labeled intermediates are prepared.

The fact that triphosphates appear to serve this system better may mean many things in view of its complexity. The most obvious is that we are dealing with an enzyme which carries out a synthesis which is a sort of hybrid of the Kornberg and Ochoa reactions and uses riboside triphosphates as the immediate precursor of the polyribonucleotide. One might expect then to find pyrophosphate exchange activity with each riboside triphosphate in the preparation. Such activities have indeed been found (Mora and Spiegelman, 1958) as shown in table 9. Failure to exhibit such exchange reactions in the preparation would have militated against the precursor hypothesis of the triphosphates. Their presence can, however, be taken at best as suggestive, since they are involved in a host of reactions generating a variety of key intermediates. It will be noted that the exchange reactions are activated by either Mn or Mg. The former, however, seems superior for the exchange with GTP. Nothing definite can as yet be stated concerning the relation of these exchange reactions to the polyribonucleotide synthesis.

The first steps toward purification of the exchange system have been taken. GTP was chosen as the best diagnostic substrate on the basis of its comparatively greater dependence on Mn. Extraction of membrane preparations with salt solutions (0.04 *M* KCl; 0.01 *M* $MnCl_2$, 0.01 *M* nucleate at

TABLE 9. *Pyrophosphate exchange in membrane fraction.*

The reaction was run at pH 6.5 buffered with Tris-maleate (0.05 *M*). Each ml contained 0.1 μM of radioactive pyrophosphate (5×10^3 cpm per mμM; and 0.3 μM of the indicated triphosphate. The reaction was stopped with trichloracetic and the extent of exchange measured by the method of Crane and Lipmann (1953). The figures represent mμM exchange in 5 minutes per 100 μg of protein.

	mμM of PP^{32} exchanged in 5 minutes		
	Mn (1×10^{-3} *M*)	Mg (1×10^{-3} *M*)	None
ATP	0.40	2.94	0
GTP	3.5	0.70	0
CTP	4.7	23.3	0
UTP	1.9	1.2	0

pH 6.5) have yielded soluble preparations capable of carrying out the GTP–PP exchange reaction at rates equivalent to the original fragments.

Until the PRN synthesizing system reported here is purified extensively, no unchallengeable statements can be made on the question of its difference from the polyribonucleotide phosphorylase. Nevertheless, it may be of interest to compare their properties as known to date. These are summarized in a comparative fashion in table 10.

The most striking differences reside in the apparent substrate and ionic requirements. Further, the present system seems to be more sensitive to orthophosphate than the PRN phosphorylase. In addition, the latter is virtually insensitive to pyrophosphate at levels which completely inhibit the system described here. This may be related to its requirement for Mn. However, the significance of the interaction with pyrophosphate is still under investigation.

The most intriguing property is its sensitivity to DNAase. The further exploration of this interesting finding may yield a clue pertinent to the relation between DNA and RNA metabolism.

TABLE 10. *Comparison of polyribonucleotide synthesizing systems of* E. coli.

Conditions	PRN-phosohorylase	Present system
Riboside triphosphates	Inactive	Required for immediate synthesis
Riboside diphosphates	Substrates	Poor synthesis always accompanied by a lag
Mg	Required as activator	Not required, inhibits
Mn	Not required; inhibits	Required as an activator
Orthophosphate (0.001 *M*)	Inhibits partially	Inhibits completely
Pyrophosphate (0.001 *M*)	No effect	Inhibits completely
DNAase	–	Inhibits

SUMMARY AND CONCLUSIONS

Starting with protoplasts, methods and environmental conditions have been devised to fractionate *E. coli* cells centrifugally into three defined components; (*a*) soluble protein; (*b*) ribonucleoprotein particles; (*c*) protoplast membranes. Examination of the behaviour of these fractions *in situ* leads to the conclusion that active synthesis of protein and RNA occurs on the membranes. The other fractions are relatively inert. The *in vitro* behaviour of these fractions with regard to protein synthesis is in agreement with this conclusion. The membrane fraction possesses more than 100 times the activity of the other two when measured in terms of the incorporation of labeled amino acids into linkages stable to hot acid. It is, furthermore, the only fraction to exhibit any evidence that it can carry out the fabrication of complete protein molecules.

The supplements required to observe optimal incorporation of amino acids in the membrane fraction suggested the presence of active nucleic acid metabolism. By including spermine in the incubation mixture it was possible to obtain extensive (10-fold) and reproducible net synthesis of polyribonucleotide. Optimal conditions for such synthesis require ribosidetriphosphates, ATP, Mn and a pH between 6.0 and 6.5. The reaction is inhibited by ortho- and pyrophosphates as well as by RNAase and DNAase. When supplemented with all four riboside triphosphates, the product formed has the properties of a polyribonucleotide strand containing a mixture of purine and pyrimidine bases.

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